



eCOMMONS

Loyola University Chicago
Loyola eCommons

Master's Theses

Theses and Dissertations

1976

The Identification and Study of the Cariogenicity of the Cultivable Anaerobic Microorganisms of the Dental Plaque of Orthodontic Patients

Robert C. Gaudry
Loyola University Chicago

Recommended Citation

Gaudry, Robert C., "The Identification and Study of the Cariogenicity of the Cultivable Anaerobic Microorganisms of the Dental Plaque of Orthodontic Patients" (1976). *Master's Theses*. Paper 2873.
http://ecommons.luc.edu/luc_theses/2873

This Thesis is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Master's Theses by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.



This work is licensed under a [Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License](https://creativecommons.org/licenses/by-nc-nd/3.0/).
Copyright © 1976 Robert C. Gaudry

THE IDENTIFICATION AND STUDY OF THE CARIOGENICITY
OF THE CULTIVATABLE ANAEROBIC MICROORGANISMS
OF THE DENTAL PLAQUE OF ORTHODONTIC PATIENTS

by

Robert C. Gaudry, D.D.S.

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science

February

1976

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to the following:

To John Vincent Madonia, D.D.S., Ph.D., my thesis advisor, for his assistance.

To Sheldon Gelbart, B.S., M.S., Ph.D., for his generous and unselfish assistance that made this work possible.

To my wife, Kathleen, for her love, devotion and persistent encouragement which enabled me to continue despite the many obstacles, and for her secretarial assistance in the preparation of this thesis.

To my wife's parents, Mr. and Mrs. Walter M. Czosnek, and to my parents, Mr. and Mrs. Thomas L. Gaudry, for their love, encouragement, and assistance.

AUTHOR'S BIOGRAPHY

Robert C. Gaudry was born in New Orleans, Louisiana, on May 22, 1944. He graduated from Jesuit High School in New Orleans in 1962, and then attended the University of New Orleans. He received the degree of Doctor of Dental Surgery in 1969, after graduating from the Dental School at Loyola University of the South. He completed a Rotating Dental Internship at Walter Reed General Hospital in 1970, and served three years at the Second Field Hospital in Bremerhaven, Germany. He has been a postgraduate student in the Loyola Orthodontic Department, and has been enrolled in the Department of Oral Biology of the Loyola Graduate School, working toward a specialty certificate in Orthodontics and a Master of Science degree in Oral Biology, respectively, since June, 1973.

TABLE OF CONTENTS

Chapter	Page
I. INTRODUCTION.....	1
II. REVIEW OF THE LITERATURE.....	3
A. Microorganisms in Disease.....	3
B. Microorganisms in Dental Caries.....	4
C. Plaque in Dental Caries.....	7
D. Acid in Dental Caries.....	8
E. Plaque and Dental Caries in the Orthodontic Patient.....	10
F. Anaerobes in Plaque.....	12
G. Summary.....	13
III. METHODS AND MATERIALS.....	14
A. Subject Selection.....	14
B. Plaque Collection.....	14
C. Isolation of Anaerobic Microorganisms.....	14
D. Identification: Gram Stain.....	16
E. Identification: Catalase Reaction.....	16
F. Identification: Gas Chromatography.....	17
G. Identification: Plaque Production.....	21
H. Identification: Acid Production.....	21
I. Materials.....	22
IV. RESULTS.....	25
V. DISCUSSION.....	33
VI. SUMMARY.....	39
VII. LITERATURE CITATIONS.....	40

CHAPTER I

INTRODUCTION

Antony van Leeuwenhoek has been called "the Father of Protozoology and Bacteriology" by Burnett and Scherp.⁽¹⁴⁾ One of the first of his many discoveries was that his mouth fluids contained "many very little living animacules"⁽¹⁷⁾ i.e. microorganisms. Later researchers also became aware of microorganisms in their struggle against man's most common disease: dental caries.⁽⁵⁹⁾ Many scientists have devoted a great deal of effort into identifying the role of microorganisms in dental caries. They discovered that in the presence of the proper carbohydrate, certain bacteria produce acids. These acids attack the enamel surface when held in contact with it by dental plaque. The plaque is formed by certain bacteria which produce extracellular polysaccharides. This combination of acid and plaque production in vivo imitates dental caries.⁽¹⁴⁾ Unfortunately, the bulk of this early research was on aerobic microorganisms. More recent research has shown that anaerobic microorganisms constitute more than half of the normal flora of plaque.^(30, 61)

The purpose of this thesis is to identify and study cultivatable anaerobic microorganisms found in plaque of orthodontic patients. The cariogenicity of these organisms will be assessed by their ability to produce acid in the presence

of certain carbohydrates and by their ability to form plaque in vitro. Only patients that have been in bands for more than six weeks were selected to assure that the oral flora had stabilized in its new environment.

CHAPTER II

REVIEW OF THE LITERATURE

A. Microorganisms in Disease.

The earliest written evidence of man's knowledge of caries dates about 5,000 B.C. It is a Sumerian text telling of "The Legend of the Worm". It told of the little worms gnawing away at the tooth and prescribed medicaments and incantations to get rid of them.⁽⁵⁶⁾ It is possible that this is not so far from the truth as was once thought.

Leeuwenhoek is conceded to have made the first observations of microorganisms in the oral cavity. Although not educated in a university, he succeeded in developing an improved lens which enabled him to become one of the leading microscopists of his day. Using only a biconvex lens, more like a simple magnifying glass than a microscope, Leeuwenhoek succeeded in observing bacteria in his own saliva and from scrapings from his teeth.⁽¹⁴⁾

Jacob Henle published a set of standards for establishing that an organism is responsible for a disease. One of his students, Robert Koch, later restated these ideas which are now known as Koch's postulates.⁽⁵⁶⁾ They are: 1) The organism must be found in all cases of the disease and its distribution must be consistent with the lesions observed; 2) The organism must be isolated in pure culture and

cultivated through a number of generations outside of the host; 3) The pure culture isolated must reproduce the typical disease when it is inoculated into the host organism; 4) It should be obtained in pure culture from this host organism.

The germ theory of disease was not generally accepted until 1882, when Koch presented a most thorough paper entitled "The Etiology of Tuberculosis".⁽⁵⁵⁾ This work overwhelmed even Virchow, the leading pathologist of the day, who had his own dualistic theory of the nature of tuberculosis.⁽¹⁷⁾

B. Microorganisms in Dental Caries.

Despite the many observations by the early scientists of bacteria in close association with teeth, their role in dental caries was generally ignored. The attitude of Pierre Fauchard, the Father of Dentistry, was typical: "The vulgar and certain authors have believed and still believe that all toothache and caries are caused by dental worms and that these worms gnaw away little by little the tissue of the bony fibers or nervous filaments."⁽²²⁾

It appears that Erdl⁽⁵⁶⁾ in 1843, was the first to implicate microorganisms in dental caries. He recommended a treatment of creosote and nitric acid to inactivate the microorganisms. In 1884, Underwood⁽⁶³⁾ demonstrated the presence of microorganisms in carious dentin but noted its absence in

normal intact dentin.

W.D. Miller, (46, 48) who studied in Koch's laboratory, has laid the foundation for our current concepts on the etiology of dental caries. His concept is known as the chemico-parasitic theory of dental caries, because it combined the then divergent views of a chemical cause with a parasitic cause for dental caries. His hypothesis stated that the decay process consisted of three stages: 1) the decalcification of the enamel which caused its complete destruction; 2) the decalcification of the dentin which left the organic matrix; 3) the destruction of the organic matrix by the microorganisms. Miller used Pasteur's discovery of the fermentation of carbohydrates to lactic acid to explain the source of acids used in the decalcification stage of dental caries. (56) The most important statement made by Miller has been found to still be true today: "The acid which affects this primary decalcification is derived from the fermentation of starches and sugar lodged in the retaining centers of the teeth." (47) Miller felt that caries was not due to any single organism, but to a variety of organisms. Today, scientists still believe that the role of microorganisms in dental caries is due to their ability to produce acid from carbohydrates.

As early as 1900, Goadby (31) had isolated a Gram-positive bacillus from carious dentin which he felt was the

cause of decay. In 1915, Gies and Kligler⁽⁴⁰⁾ isolated a Lactobacillus and demonstrated that it produced acid. In 1926, Bunting et al found that there was a strong correlation between the presence of Lactobacillus acidophilus in the caries susceptible mouth, and its absence in caries-free mouth. Jay and Voorhees⁽³⁵⁾ found that the presence of Lactobacillus was so important that it could be used to predict future caries activity. Numerous dental schools and practitioners still use the Lactobacillus count as a measure of caries activity in their preventive dentistry program. Florestano,⁽²⁶⁾ in 1942, isolated acidogenic Streptococci and Staphylococci from patients. He found that the patients with more caries had greater numbers of these bacteria. He suggested that these microorganisms might have as an important a role in dental caries as Lactobacillus.

In 1954, Orland⁽⁵³⁾ could not produce caries in germ-free rats, although they were on caries-inducing diet. Normal rats did develop caries while on this same diet. Later, Orland and others⁽⁵²⁾ did succeed in producing caries in germ-free rats by inoculating them with Enterococci.

In 1960, Keyes⁽³⁸⁾ published his important work demonstrating that under certain conditions caries-inactive hamsters would develop caries when inoculated with a certain Streptococci. Fitzgerald and workers⁽²⁵⁾ confirmed the transmission

of caries in gnotobiotic rats. In both experiments, caries-inactive or gnotobiotic animals were fed a normally caries-inducing diet. In neither case did the animals develop caries unless certain Streptococci were introduced into the animals' environment. The caries produced by the Streptococci was very similar to that of the control animal on the caries-inducing diet.

Keyes⁽³⁹⁾ summarized what he felt demonstrated "the infectious and transmissible nature of experimental caries: 1) the carious flora must be acquired; 2) the teeth must be susceptible (young); 3) the diet must be caries promoting."

In 1966, Fitzgerald and others⁽²⁴⁾ repeated this earlier experiment on gnotobiotic rats. This time they succeeded in producing caries in the rat using Lactobacillus. At about the same time, Krasse⁽⁴²⁾ found that the Streptococcus that caused caries in humans was very similar to the strain that produced caries in the hamster. Krasse, in fact, succeeded in producing caries in the hamsters with the Streptococcus that he had isolated from humans with active caries.

Edwardsson⁽²⁰⁾ isolated forty-nine strains of Streptococci from humans. He found them all to be very similar and all produced rampant caries in the hamster.

C. Plaque in Dental Caries.

Miller⁽⁴⁷⁾ implicated bacteria in caries as early as

1890. G.V. Black⁽⁸⁾ also joined in the early conviction of plaque as the agent in caries. Bibby⁽⁷⁾ in 1940, noted the ability of the filamentous organisms to adhere to smooth surfaces. It is felt that the filamentous organisms form a mesh which traps the smaller free-floating bacteria and acts as an anchor to allow them to adhere to the tooth. Since this time, other species have been shown to form plaque by many researchers. (27, 29, 36, 49) In 1958, Arnim⁽²⁾ wrote a very thorough indictment of plaque. In this article, he gave an excellent description of plaque. Arnim's work is now part of the foundation of "plaque control" in modern preventive dentistry. There is now general agreement that caries begins beneath the plaque.⁽⁵⁹⁾ The mechanism of how this occurs is still not fully understood, although a great deal has been learned about the process.⁽¹⁴⁾ We do know that plaque will begin to adhere to the tooth in 24-48 hours unless it is removed.⁽²⁾ The production of plaque has been shown to be necessary for caries induction.⁽²⁷⁾ In the presence of the appropriate carbohydrate, the plaque will cause a drop in the pH.⁽³⁶⁾ Finally, this entire process can cause caries, in vitro, in experimental animals, and in man.

D. Acid in Dental Caries.

Miller⁽⁴⁸⁾ noted that litmus paper in contact with a

carious lesion eventually turned red showing the presence of acid. Gibbons and Socransky⁽²⁹⁾ suggested that intracellular polysaccharides stored by microorganisms in dental plaque may be metabolized later. This would suggest that these microorganisms would not only produce acid when carbohydrates were present in their environment, but that they would continue to produce acid for a long period of time as the stored polysaccharides were degraded. Jordan and Keyes⁽³⁶⁾ developed a mechanism to grow plaque on teeth and wires. Where the teeth were unprotected by wax, lesions resembling incipient caries developed. To further prove their point, the same mechanism was used to develop plaque on a pH meter probe. The probe showed a drop in pH after it was covered with plaque.

Bowen et al⁽¹⁰⁾ fasted monkeys for 17 hours, until the pH on carious lesions in their mouths was 7 or above. After application of sugar solutions to the carious area, the pH dropped rapidly. This group attributed caries to the acidogenic process.

Burnett and Scherp⁽¹⁴⁾ have said that "we know of no way as yet to initiate the decomposition of enamel, cementum, and dentin except by decalcification." The same authors⁽¹²⁾ also found that aciduric bacteria were associated with the shallow carious lesion in enamel. They felt that decalcification was the "prime activity" in the initial stages. In

another study,⁽¹³⁾ acidogenic bacteria isolated from carious lesions were cultured and used to decalcify dentin. Lactobacilli decalcified dentin up to 75% in this study.

It is obvious now that most researchers in the field agree with Gibbons and Banghart⁽²⁷⁾ that the plaque is the mechanism by which the acid is localized on the tooth surface and where during the initial stage of caries, the decalcification takes place. Many different species of microorganisms participate in the initiation of caries and the carious lesion.

E. Plaque and Dental Caries in the Orthodontic Patient.

It was not long after the invention of the fixed orthodontic appliance that dentists became aware that in some patients the caries rate rose. Noyes⁽⁵¹⁾ in an early investigation (1937) found that the etching beneath orthodontic bands was very similar to the initial carious lesion. He recognized that the orthodontic appliances gave the microorganisms a greater chance of survival. However, he did not answer the question as to whether there was an increased incidence of caries among orthodontic patients.

In 1941, Burrill⁽¹⁵⁾ attempted to answer this question in a study. The result was inconclusive with a group of highly caries-susceptible patients becoming less susceptible, the low caries-susceptible patients becoming more susceptible and

an intermediate group divided both ways.

Owen⁽⁵⁴⁾ ran salivary Lactobacillus counts before and after appliances were inserted and found that there was a definite increase after appliance insertion. Dolce⁽¹⁸⁾ found an increase in the incidence of caries in the orthodontic patient; he attributed this to poor oral hygiene.

Bloom and Brown⁽⁹⁾ did an extensive study on the oral microbial flora before and after banding. Only the Lactobacillus population showed a statistically significant increase in population. Adams⁽¹⁾ also found a significant increase in the number of Lactobacilli; after banding, his study indicated a concurrent increase in caries which decreased after the bands were removed.

Balenseifen⁽⁵⁾ made a study of plaque in the orthodontic patient before and after banding. He used an exploring (antimony-silver) tip electrode to make in the mouth measurements of the pH of the plaque. His study showed a definite increase of the acidity of the plaque. He also found a forty percent increase in the carbohydrate content of the plaque. The Lactobacillus, Streptococcus mitis, and Streptococcus salivarius were also found to have increased in significant amounts. Riggs⁽⁵⁷⁾ in another before and after banding study of orthodontic patients did not find any increase of the acidogenesis of the bacteria. However, he did find a significant

increase in the numbers of bacteria and attributed the increased caries potential of orthodontic patients to the increased numbers of organisms.

Bloom and Brown⁽⁹⁾ did a survey of seven microbial populations in plaque before and after banding. Only Lactobacilli had increased significantly after banding.

F. Anaerobes in Plaque.

Jay,⁽³⁴⁾ in 1927, isolated an anaerobic microorganism from carious lesions, first indicating the role anaerobes may have in caries. Gibbons, Socransky and workers^(30, 61) in 1963 did a baseline study on anaerobes in plaque. They found that the majority of bacteria in plaque were obligate anaerobes. The Bloom and Brown study⁽⁹⁾ supported their findings. This study also found a numerical increase in the numbers of anaerobes after banding, but this was not shown to be statistically significant.

In a recent study, Loesche and Syed⁽⁴⁴⁾ found the ratio of anaerobic to aerobic microorganisms in carious plaque to be eleven to one. The same ratio for carious dentin was seven to one; in addition, the pH in carious areas of the plaque was found to be lower than the non-carious. The ratio for saliva is ten to one anaerobic to aerobic microorganisms.⁽⁵⁸⁾

G. Summary.

This review has shown that previous research has attempted to prove that the initial stage of dental caries is due to the destruction of the enamel by acids. It has been demonstrated that the production of these acids is due to certain strains of microorganisms. Early research only involved the aerobic organisms; more recent work has shown the importance of the anaerobic organisms. Much work has also been done to show the profound changes on the oral microbial population affected by the presence of orthodontic appliances. The present investigation is an attempt to identify the strains of anaerobic microorganisms found in the plaque of orthodontic patients and their acid-producing potential in the presence of certain carbohydrates.

CHAPTER III

METHODS AND MATERIALS

A. Subject Selection.

Twelve patients, who had been wearing fixed orthodontic appliances for a minimum of six weeks, were selected at random from the Orthodontic Clinic at the Loyola University School of Dentistry. These patients were selected without regard to age, sex, race, malocclusion, or type of orthodontic technique being used.

B. Plaque Collection.

The patients were using their normal oral home care procedures, but they were asked not to brush their teeth for two hours prior to the time the plaque sample was taken. The site chosen for the sampling was the bucco-gingival surface of the upper first permanent molar. The plaque sample was taken with a sterile wire probe made of non-oxidizing stainless steel wire. The sample was transferred directly to two milliliters of sterile normal saline.

C. Isolation of Anaerobic Microorganisms.

The plaque was mixed with the saline on a vortex mixer until all of the particles were evenly disbursed. This suspension was serially diluted by taking 1.0 milliliter of the ori-

ginal suspension and adding it to 9.0 milliliters of sterile saline. The dilution was also mixed on a vortex mixer. This dilution procedure was carried out until dilutions of 10^{-6} , 10^{-7} , and 10^{-8} were obtained. These dilutions were chosen because they were recommended by the U.C.L.A. Anaerobic Bacteriology Manual.⁽⁶²⁾

The initial isolation was done on pre-reduced anaerobic sterile (hereafter abbreviated PRAS) plates of a modified brain-heart infusion agar (hereafter abbreviated BHIA) and BHIA with 5% defibrinated sheep blood (hereafter abbreviated Blood). One tenth of a milliliter of the final dilution was spread evenly across the face of the plate with a sterile bent glass rod. One plate of PRAS BHIA and Blood was made for each dilution of 10^{-6} , 10^{-7} , and 10^{-8} . The plates were incubated anaerobically in a GasPak Jar^{*} at 37°C for ten days.

The reduction of the atmosphere in the GasPak Jar was confirmed by the presence of a strip of moist filter paper impregnated with methylene blue. The methylene blue changed from a blue color when oxidized, to colorless when reduced. Both BHIA and Blood plates contained resazurin which changed from pink to colorless when reduced. These indicators assured complete removal of oxygen from the atmosphere of the GasPak

* BioQuest, Box 243, Cockeysville, MD 21030

Jar. The GasPak catalyst cartridge was regenerated after each use by heating to 160°C for $1\frac{1}{2}$ to 2 hours in a drying oven.

After the incubation period, the jar was opened and the plates were examined. At least one of each type of colony was inoculated into the culture media. Only separate and distinct colonies were inoculated into the culture media to avoid a mixed culture. If the original colony was large enough, a smear was made at the same time which was later Gram stained.

Those colonies which were picked for subculture were marked and the plates were incubated aerobically for an additional three days at 37°C . The colonies which continued to grow or survived the exposure to air were not considered to be obligate anaerobes. Their cultures were discarded.

D. Identification: Gram Stain.

The smear made at the time that the original colony was picked was Gram stained to check the purity of the original isolate. The Gram stain was also used to establish cell morphology, the Gram reaction and the presence of spores.

E. Identification: Catalase Reaction.

PRAS BHIA and Blood plates were divided into quarters and each isolate was streaked on a quarter of a PRAS BHIA or

Blood plate depending on which media it was isolated. Isolates that originated from a BHIA plate were streaked on that medium and similarly for those from Blood plates. The streak plates were incubated anaerobically for five days at 37°C. After this period, the plates were exposed to air for thirty minutes. Each isolate was flooded with 3% hydrogen peroxide. The evolution of bubbles was considered a positive reaction.

F. Identification: Gas Chromatography.

The colonies picked for culture and identification were subcultured in BBL^{*} Chopped Meat and/or Fluid Thioglycollate Medium; both media were supplemented with hemin and menadione. Chopped Meat Medium hereafter will be abbreviated CMG (Chopped Meat Glucose) and Fluid Thioglycollate Medium will be abbreviated Thioglycollate. As only the dehydrated CMG was available, its use was discontinued after the sixth patient because better growth was obtained in the Thioglycollate.

After inoculation, the tops to the tubes were left slightly unscrewed so that the oxygen introduced during inoculation would be removed by the GasPak apparatus. The reduction of the Thioglycollate was indicated by the loss of the pink color in the upper layer of the media.

* Baltimore Biological Laboratories, Cockeysville, MD 21030

Two tubes of Thioglycollate from each colony were inoculated. One tube was used as a master culture and the other was used in the identification of the fermentation products with the gas chromatograph.

A forty-eight hour culture had been recommended for use in the gas chromatograph, according to the outline given for use of this instrument by clinical laboratories.⁽²¹⁾ Preliminary studies showed that insufficient amounts of the acid and alcohol products were available for detection. Therefore, cultures were incubated as before, but until sedimentation or turbidity indicated the presence of adequate growth. This was usually no more than five to ten days.

The culture to be examined was first acidified (to pH 2 or less) by the addition of about 0.2 milliliters of 50% aqueous sulfuric acid per 12 milliliters of culture. The salt forms of the fermentation acids ($R-COONa$) are soluble in water, but not ether. At pH 2 or less, these fermentation acids are in the free acid form ($R-COOH$) which are soluble in water and even more so in ether.

To obtain the ether soluble products about two milliliters of acidified culture was pipetted into a small test tube. The remainder of the acidified culture was saved for the identification of the methylated products if needed later. One milliliter of ethyl ether was added to the small test tube and

mixed thoroughly with the acidified culture. In most cases, it was necessary to centrifuge the mixture to break the ether-culture emulsion.

The ether layer was then pipetted into another test tube without including any of the water mixture. A small amount of anhydrous calcium chloride, about half the volume of the ether, was added to the test tube. The tube was stoppered and mixed thoroughly. The anhydrous calcium chloride removed the dissolved water from the ether. This was very important as a large water peak on the chromatograph could cover the first few acid peaks. Fourteen microliters of the anhydrous ether extract were injected into the number one column of the gas chromatograph.

A Dohrmann^{*} Anaerobic Bacteriology Analyzer was used for the gas chromatography; it has a built-in recording unit. The operating conditions for the gas chromatograph were the same as those recommended by the V.P.I. Manual.⁽³³⁾ Separate columns were used for the ether extracted and the chloroform extracted methylated fermentation products.

Identification of the methyl derivatives was done only if indicated by the V.P.I. Manual.⁽³³⁾ One milliliter of the original acidified culture was transferred into a test tube. Two milliliters of methanol and 0.4 milliliters of 50% aqueous

* Dohrmann Division-Envirotech Corporation, Mountain View, CA

sulfuric acid was added to the acidified culture. The tube was stoppered and heated to 55°C for thirty minutes. One milliliter of water and 0.5 milliliter of chloroform was added. The tube was restoppered and mixed. The tube was centrifuged to break the emulsion and the chloroform layer was drawn directly into the injection syringe. Fourteen microliters of the chloroform layer were injected into the number two column.

The chromatographs from the cultures were compared to those obtained from standard solutions containing one milliequivalent of acid or alcohol per 100 milliliters of solution. Only the presence or absence of the acid or alcohol in the culture was important. The chromatographs from the cultures were compared to those given in the V.P.I. Manual⁽³³⁾ to help establish the identity of the microorganism.

The gas chromatograph used in this study was unable to discriminate the presence of formic acid in amounts less than 10 milliequivalents/100 milliliters. However, the V.P.I. group⁽³³⁾ had a similar problem and it was noted in the manual. This did not present a problem in identification of any of the microorganisms.

The uninoculated Thioglycollate was also run through the gas chromatographic analysis. A small amount of acetic acid was found to be present and its presence was corrected for when interpreting data from the cultures.

G. Identification: Plaque Production.

The production of plaque was determined by the ability of the microorganism to adhere to and form plaque on a wire in the presence of glucose.⁽⁴⁹⁾ A sterile piece of .021 X .025 inch stainless steel wire was aseptically placed in the growing Thioglycollate culture. After five days, it was transferred to a sterile tube of Thioglycollate. At the end of five days, if there was any growth on the wire, it was rated as positive for plaque formation in the presence of glucose.

H. Identification: Acid Production.

The fermentation of carbohydrates to form acids was judged by the ability of the microorganisms to lower the pH of BBL Cysteine Trypticase Agar containing 5% carbohydrate. The carbohydrates used were glucose, sucrose, maltose, lactose, manitol, and sorbitol. (These media were abbreviated CTA glucose, etc.) Glucose, sucrose, lactose, and maltose were chosen because they are sugars found commonly in the diet. Manitol and especially sorbitol are common sugar alcohols used in dietetic foods. CTA contains phenol red, a pH indicator which is red at pH 8.5 or above, and yellow at pH 6.5 or below.

The CTA tubes were inoculated with an .025 inch non-oxidizing stainless steel wire to the depth of the tube. Particular care was taken to see that there were a few drops of

culture on the wire to assure a heavy inoculation of the CTA. Due to a lack of equipment, the CTA could not be pre-reduced, nor were any special growth enhancers included in the media. Sterile CTA tubes were incubated with the samples as a control; no color change due to the anaerobic atmosphere was seen. The tubes were incubated anaerobically with their tops loose in a GasPak Jar under the usual conditions.

At the end of five days, the results were recorded. Those tubes with no growth were recorded as NG. Tubes that contained growth, but did not change color from red to yellow were recorded as negative; those that did produce a color change were recorded as positive. The results of the CTA-carbohydrate tests were used to confirm the identification of the microorganisms.

I. Materials.

1. BHIA (Brain-Heart Infusion Agar - Supplemented):

Brain-heart infusion broth, dehydrated	3.7 g.
Yeast extract	0.5 g.
Cysteine	0.05 g.
Agar	2.5 g.
Distilled water	100.0 ml.
Resazurin solution	0.4 ml.

Boil and autoclave at 15 p.s.i. for 15 minutes. Cool to 55°C. Add:

Sterile injectable menadione (Vitamin K)	0.25 mg.
Hemin solution	1.0 ml.

2. Resazurin solution:

Resazurin	11	mg.
Distilled water	44	ml.

3. Hemin solution:

Dissolve 50 milligrams of hemin in one milliliter of one normal sodium hydroxide; make to 100 milliliters with distilled water. Autoclave at 120°C for 15 minutes.

4. Blood (BHIA with 5% Blood):

Make up BHIA as above; after it has been cooled to 55°C, add five milliliters of fresh sterile defibrinated sheep blood for each 100 milliliters of media.

5. PRAS BHIA and Blood Plates:

After the above media have been poured and they have cooled sufficiently to invert, they should be immediately placed in a GasPak Jar. Three Gas Generating Paks should be placed in the Jar with 10 milliliters of water each. The catalyst must be fresh or recently regenerated. If this is done, then the resazurin in the BHIA plates should turn colorless and the Blood plates should turn a deep dark red in less than 48 hours at room temperature.

6. CMG (Chopped Meat Broth - Supplemented):

Use dehydrated Chopped Meat Broth with glucose. Follow manufacturers' directions and add to each 100 ml.

Menadione (sterile injectable Vitamin K fluid)	0.25 mg.
Hemin solution	1.0 ml.

7. Thioglycollate (Thioglycollate Medium - Supplemented):

Use dehydrated Fluid Thioglycollate Medium with glucose.

Follow manufacturers' instructions and add

Hemin solution	1.0 ml.
Menadione (sterile injectable Vitamin K fluid)	0.25 mg.

The liquid media were dispensed in tubes with screw tops. The tops were screwed down tightly before autoclaving. The autoclave was allowed to cool overnight instead of the usual exhaust cycle in order to prevent the tubes from exploding. This process excluded the bulk of the air from the tubes. Although this was not equivalent to reduction under CO₂, it was as close as possible with the limited equipment available. This was a modification of the method used by Attebery and Finegold.⁽³⁾

CHAPTER IV

RESULTS

As a result of this investigation, twenty-three obligately anaerobic microorganisms were isolated from the plaque of twelve orthodontic patients (Table 1). All of these microorganisms were identified to genus and eighteen were tentatively identified to species.

The unknown cultures were first tentatively identified by their Gram reaction, morphology, catalase reaction and fermentation products (Table 2). The volatile acids, alcohols, and methylated acids were identified by the gas chromatograph. The products of the unknown cultures with other characteristics were compared to the characteristics of known cultures. Cultures having the same traits as the known strain were considered identified for the purpose of this investigation.

Thirteen of these microorganisms were Gram-positive cocci, eight were Gram-positive rods, and one was a Gram-negative rod. The microorganisms isolated were: Propionibacterium acnes (two strains isolated), Arachnia propionica (two strains), Lactobacillus sp. (one strain), Eubacterium limosum (one strain), Eubacterium lentum (two strains), Bacteroides oralis (one strain), Peptostreptococcus intermedius (two strains), Peptostreptococcus micros (five strains), Peptostreptococcus productus (two strains), Peptostreptococcus

sp. (four strains) and Peptococcus constellatus (one strain).

The cariogenic potential of these microorganisms was assessed in vitro by the ability to ferment carbohydrate and produce acid and also by the ability to form plaque on an orthodontic wire (Table 3).

Seven of the microorganisms fermented sugar or sugar alcohols in CTA to produce acid. The sugars used were glucose, sucrose, maltose, and lactose, while manitol and sorbitol were the sugar alcohols used. Those organisms found to have varying abilities to ferment carbohydrates were: Peptostreptococcus intermedius, Peptococcus constellatus, Propionibacterium acnes, Eubacterium limosum, Peptostreptococcus sp. and Peptostreptococcus micros.

One of the microorganisms, Lactobacillus sp., was able to form plaque in vitro on an orthodontic wire in Thioglycollate medium with glucose.

TABLE 1

<u>Patient</u>	<u>Age</u>	<u>Sex</u>	<u>Treatment Time - Months</u>	<u>Microorganism Isolated</u>
1	15	F	3	<u>Peptostreptococcus intermedius</u> Unable to subculture: two
2	15	F	3	Unable to subculture: four
3	15	F	18	<u>Peptococcus constellatus</u> Unable to subculture: five
4	12	M	6	<u>Propionibacterium acnes</u> <u>Arachnia proprionica</u>
5	14	M	36	<u>Peptostreptococcus</u> sp. <u>Peptostreptococcus</u> sp.
6	12	M	6	<u>Eubacterium limosum</u> <u>Peptostreptococcus</u> sp. <u>Lactobacillus</u> sp. <u>Peptostreptococcus intermedius</u>
7	13	F	24	<u>Peptostreptococcus micros</u> <u>Peptostreptococcus micros</u>
8	13	M	6	<u>Peptostreptococcus micros</u> <u>Bacteroides oralis</u>
9	15	F	17	<u>Peptostreptococcus micros</u>
10	14	F	18	<u>Eubacterium lentum</u> <u>Peptostreptococcus</u> sp. <u>Propionibacterium acnes</u>
11	17	F	7	<u>Peptostreptococcus productus</u> <u>Peptostreptococcus productus</u> <u>Arachnia propionica</u>
12	13	M	20	<u>Eubacterium lentum</u> <u>Peptostreptococcus micros</u>

TABLE 2: CRITERIA FOR IDENTIFICATION

	Patient	Gram Stain	Morphology	Catalase	Fermentation Products
<u>Propionibacterium acnes</u>	4	+	R	-	PA
<u>Propionibacterium acnes</u>	10	+	R	-	PA
<u>Arachnia propionica</u>	4	+	R	-	AP
<u>Arachnia propionica</u>	11	+	R	-	AP
<u>Lactobacillus</u> sp.	6	+	R	-	La
<u>Eubacterium limosum</u>	6	+	R	-	A
<u>Eubacterium lentum</u>	10	+	R	-	A
<u>Eubacterium lentum</u>	12	+	R	-	A
<u>Bacteroides oralis</u>	8	-	R	-	A
<u>Peptostreptococcus intermedius</u>	1	+	C	-	a
<u>Peptostreptococcus intermedius</u>	6	+	C	-	a
<u>Peptostreptococcus micros</u> A	7	+	C	-	A
<u>Peptostreptococcus micros</u> B	7	+	C	-	A
<u>Peptostreptococcus micros</u>	8	+	C	-	A

TABLE 2: CONTINUED

	Patient	Gram Stain	Morphology	Catalase	Fermentation Products
<u>Peptostreptococcus micros</u>	9	+	C	-	A
<u>Peptostreptococcus micros</u>	12	+	C	-	A
<u>Peptostreptococcus productus</u> A	11	+	C	-	A
<u>Peptostreptococcus productus</u> B	11	+	C	-	A
<u>Peptostreptococcus</u> sp. A	5	+	C	-	A
<u>Peptostreptococcus</u> sp. B	5	+	C	-	A
<u>Peptostreptococcus</u> sp.	6	+	C	-	A
<u>Peptostreptococcus</u> sp.	10	+	C	-	Abiv
<u>Peptococcus constellatus</u>	3	+	C	+	a

TABLE 3: CARIOGENIC POTENTIAL

	Glucose	Sucrose	Maltose	Lactose	Sorbitol	Manitol	Plaque Formation
<u>Propionibacterium acnes</u>	+	-	-	-	-	-	-
<u>Propionibacterium acnes</u>	NG	NG	NG	-	NG	NG	-
<u>Arachnia propionica</u>	NG	NG	NG	NG	NG	NG	-
<u>Arachnia propionica</u>	-	NG	NG	NG	-	-	-
<u>Lactobacillus sp.</u>	NG	NG	NG	NG	NG	NG	+
<u>Eubacterium limosum</u>	+	+	+	+	+	+	-
<u>Eubacterium lentum</u>	NG	NG	NG	NG	NG	NG	-
<u>Eubacterium lentum</u>	-	NG	NG	NG	NG	NG	-
<u>Bacteroides oralis</u>	NG	NG	NG	NG	NG	NG	-
<u>Peptostreptococcus intermedius</u>	+	+	+	+	-	-	-
<u>Peptostreptococcus intermedius</u>	+	+ ^w	+ ^w	+	-	+	-
<u>Peptostreptococcus micros</u> A	NG	NG	NG	NG	NG	NG	-
<u>Peptostreptococcus micros</u> B	NG	NG	NG	NG	NG	NG	-
<u>Peptostreptococcus micros</u>	NG	NG	NG	NG	NG	NG	-
<u>Peptostreptococcus micros</u>	NG	NG	NG	NG	NG	NG	-

TABLE 3: CONTINUED

	Glucose	Sucrose	Maltose	Lactose	Sorbitol	Manitol	Plaque Formation
<u>Peptostreptococcus micros</u>	-	-	-	-	-	-	-
<u>Peptostreptococcus productus</u> A	NG	NG	NG	NG	NG	NG	-
<u>Peptostreptococcus productus</u> B	NG	NG	NG	NG	NG	NG	-
<u>Peptostreptococcus</u> sp. A	NG	NG	NG	NG	NG	NG	-
<u>Peptostreptococcus</u> sp. B	NG	NG	NG	NG	NG	NG	-
<u>Peptostreptococcus</u> sp.	+	+	+	+	+	+	-
<u>Peptostreptococcus</u> sp.	NG	NG	NG	NG	NG	NG	-
<u>Peptococcus constellatus</u>	+	+	+	-	+	+	-

EXPLANATION OF SYMBOLS

+	positive reaction
+ ^w	weak positive reaction
-	negative reaction
R	rod
C	coccus
A	major acetic acid peak
L	major lactic acid peak
P	major pyruvic acid peak
a	minor acetic acid peak
b	minor butyric acid peak
iv	minor isovaleric peak
NG	no growth

The fermentation products are listed in order of importance.

CHAPTER V

DISCUSSION

From our earliest history, man has indicated an interest in the etiology of caries.⁽⁵⁶⁾ Although we often forget it, man has been aware, at least since the time of Leeuwenhoek, that microorganisms are in intimate contact with his teeth.⁽¹⁴⁾ However, dentists have not always been aware of the importance of microorganisms in dental caries.⁽²²⁾

W.D. Miller^(46, 47, 48) focused our attention on the role of microorganisms in acid production. Most researchers today still agree that caries is initiated by the decalcification of the enamel by acid-forming bacteria. Burnett and Scherp⁽¹³⁾ demonstrated that microorganisms isolated from carious lesions were able to decalcify dentin up to 75% by producing acid. What was not understood was why the acid was not diluted and carried away by the saliva.

About the same time as Miller was doing his work, Black⁽⁸⁾ called for the removal of the plaque on teeth as a means of decreasing the incidence of caries. Then in 1958, Arnim,⁽²⁾ one of the pioneers of preventive dentistry, began a campaign against plaque which continues today.

Through the efforts of men such as Bibby⁽⁷⁾ we became aware of the interrelationships of the different microorganisms. He felt that the filamentous bacteria, although not acidogenic

themselves, presented a ready matrix to the unattached acidogenic bacteria which allowed them to settle on the tooth surface.

Gibbons and Socransky⁽²⁹⁾ first explained the production of plaque as a food storage mechanism which also allowed the microorganisms to continue to produce acid over a long period of time. In a later study, Gibbons and Banghart⁽²⁷⁾ felt that the extracellular dextran was the mechanism by which acid by-products were localized against the tooth surface. Jordan and Keyes⁽³⁶⁾ confirmed this by growing plaque in vitro on both teeth, wires, and a pH meter probe. In this investigation, they produced incipient carious lesions on the part of the tooth that was covered by the plaque. They also demonstrated a drop in pH under the plaque using the pH meter probe covered with plaque.

The results of these previous investigations support the theory that plaque is the mechanism by which caries is initiated on the tooth surface. In 1960, Fitzgerald and workers⁽²⁵⁾ demonstrated that a single strain of Streptococci was capable of producing caries in the gnotobiotic rat. Thus one microorganism was capable of producing both the plaque and the acid necessary to initiate caries. Fitzgerald, Jordan, and Archad⁽²⁴⁾ repeated this experiment with a variety of Lactobacillus acidophilus and were able to produce caries in

the gnotobiotic rat also.

Keyes⁽³⁹⁾ felt that besides the caries-producing microorganism, the diet must also be caries-inducing. There have been a number of studies^(6, 64, 65) supporting the argument that carbohydrates, especially sugars, increase the incidence of caries. Bowen et al⁽¹⁰⁾ demonstrated experimentally a drop in pH in carious lesions after sugar solutions were applied to them. Krasse⁽⁴¹⁾ found that sucrose caused an increase in the number of caries-inducing Streptococci and increased the incidence of caries.

Owen⁽⁵⁴⁾ found an increase in bacterial counts after banding in the orthodontic patient. Bloom and Brown⁽⁹⁾ in a study of the oral microbial flora found an increase in the number of Lactobacilli after banding. The same investigators found that the anaerobic microbial population was the most numerous. Adams,⁽¹⁾ Balenseifen,⁽⁵⁾ and Riggs⁽⁵⁷⁾ all found increased numbers of aerobic microorganisms after banding. Riggs attributed the decrease in pH of the plaque after banding due to the increased numbers of microorganisms. Since the ratio of anaerobic microorganisms to aerobic microorganisms in plaque is eleven to one⁽⁴⁴⁾ before banding, it would be reasonable to assume the ratio would remain the same after banding. Bloom and Brown in their study did find that the ratio remained about the same.

Some of the anaerobic microorganisms identified by this present investigation were capable of producing acid in the presence of carbohydrates. One of the microorganisms, a Lactobacillus, also was able to produce plaque in vitro.

These capabilities are most important in relation to the cariogenic potential of the microorganisms. This author has attempted to demonstrate through previous investigative efforts, the importance of the ability to form plaque and to produce acid from carbohydrates.

In the past, most of the research has concentrated on linking a specific microorganism with caries production. However, the fact that the mouth contains a mixed microbial population is often completely overlooked. Miller^(46, 47, 48) felt that caries was a mixed infection and not due to a single microorganism. It is possible the caries in vivo may be caused by one microorganism producing plaque and another producing acid. It would seem even more probable that many microorganisms are involved in both processes. Burnett and Scherp⁽¹²⁾ found that the microbial flora did change as the carious lesion progressed from the enamel to the dentin.

Another often overlooked item is that as the plaque is built up on the tooth, the deeper layers will become progressively anaerobic as the oxygen is depleted by the microorganisms. This would allow the anaerobic microorganisms to proliferate

and their role in plaque and acid production is probably greater than previously thought. At one time, this phenomenon of aerobic microorganisms providing an anaerobic atmosphere was used by Fortner⁽⁶⁶⁾ to culture anaerobic microorganisms. Fortner used a heavy culture of an aerobic microorganism such as Serratia marcescens on one agar plate which was sealed against a plate containing the anaerobic microorganism to be cultured.

Because the mouth contains a mixed microbial flora, some microorganisms may provide growth enhancing factors which cannot be duplicated in the laboratory. Certain bacteria such as Bacteroides melaninogenicus ss. asaccharolyticus are known to require media supplements such as menadione (Vitamin K) and hemin. The difficulty in isolating the more fastidious organisms has been recognized by Dwyer and Socransky⁽¹⁹⁾ who found B. melaninogenicus in only three out of five of their samples. Kelstrup also had difficulty in isolating B. melaninogenicus from patients.⁽³⁷⁾ Both menadione and hemin were included in the BHIA Blood and Thioglycollate media. Unfortunately, since we do not know all of the growth requirements of the more fastidious microorganisms, no single medium is satisfactory for their isolation.

Martin⁽⁴⁵⁾ found a significant increase in the number of anaerobic microorganisms isolated from PRAS media compared

to non-PRAS media. Because of this, the BHIA and Blood plates were pre-reduced. However, previous investigators^(32, 43) have shown that most obligate anaerobes can tolerate a brief exposure to oxygen.

CTA does not contain specific supplements, e.g. hemin and menadione, nor was it possible to pre-reduce the Thio-glycollate and CTA. The CTA was used as it is a test for the utilization of a single carbohydrate source by a microorganism. However, most of the air was driven off during the autoclaving and the screw caps prevented most of it from returning. The organisms were inoculated very heavily into the media and incubated in an anaerobic atmosphere. Collee et al⁽¹⁶⁾ felt that for most anaerobic purposes that the GasPak apparatus was adequate.

Four sugars and two sugar alcohols were used as the fermentable carbohydrates. Sugars have long been implicated in caries, but the use of sugar alcohols in man's diet is more recent. Both manitol and sorbitol can be fermented to produce acid. Sorbitol is commonly used as a dietary substitute.

Plaque production in the presence of any carbohydrates except glucose was not investigated. Admittedly, sucrose is required for plaque production by Streptococcus;⁽³⁶⁾ however, Lactobacillus can form plaque from glucose alone. It was not within the scope of this work to investigate all carbohydrates.

CHAPTER VI

SUMMARY

This investigation has isolated anaerobic microorganisms from the dental plaque of orthodontic patients and demonstrated that they have cariogenic potential. The cariogenic potential was demonstrated by the in vitro ability to form plaque or acid from carbohydrates.

This investigator feels that the importance of the role of the anaerobic microorganisms in caries has been overlooked. As the plaque is built up by the aerobic microorganisms, it is theorized that the deeper layers become progressively more anaerobic. Thus, the anaerobic microorganisms become more important in the initiation of caries as the plaque ages and becomes thicker.

CHAPTER VII

LITERATURE CITATIONS

1. Adams, R.J. The Effects of Fixed Orthodontic Appliances on the Cariogenicity, Quantity and Microscopic Morphology of Oral Lactobacilli. J. Oral Med. 22:88-98, 1967.
2. Arnim, S.S. Thoughts Concerning Cause, Pathogenesis, Treatment and Prevention of Periodontal Disease. J. Periodont. 29:217, 1958.
3. Attebery, H.R., and Finegold, S.M. Combined Screw-Cap and Rubber-Stopper Closure for Hungate Tubes. Appl. Microbiol. 18:558-561, 1969.
4. Back, E.N. Report of "Orthodontic Children" Covering a Period of Twenty-five Years. A.J.O. 40:83-108 (Feb.), 1954.
5. Balenseifen, J.W. A Study of Dental Plaque in Orthodontic Patients. Master's Thesis, Loyola University, 1968.
6. Bibby, B.G. Effect of Sugar Content of Foodstuffs on Their Caries-Producing Potentialities. J.A.D.A. 51:293-306, 1955.
7. Bibby, B.G. Studies on Dental Caries. Tufts D. Outlook, 14:4, 1940.
8. Black, G.V. Susceptibility and Immunity to Dental Caries. D. Cosmos, 41:826, 1899.
9. Bloom, R.H., and Brown, L.R. A Study of the Effects of Orthodontic Appliances on the Oral Microbial Flora. Oral Surg., Oral Med., and Oral Path. 17:658, 1964.
10. Bowen, W.H., Eastoe, J.E., and Cock, D.J. The Effect of Sugar Solutions on the pH of Plaque in Caries Active Monkeys. Arch. Oral Biol. 11:833-837, 1966.
11. Bunting, R.W., Nickerson, G., Hard, D.G. Further Studies of the Relation of Bacillus acidophilus to Dental Caries. D. Cosmos 68:931, 1926.

12. Burnett, G.W., and Scherp, H.W. The Distribution of Proteolytic and Aciduric Bacteria in the Saliva and in the Carious Lesion. *Oral Surg.* 4:469-497, 1951.
13. Burnett, G.W., and Scherp, H.W. The Effects on Dentin of Proteolytic and Acidogenic Bacteria Isolated from Carious Lesions. *J. Dent. Res.* 32:46-56, 1953.
14. Burnett, G.W., and Scherp, H.W. Oral Biology and Infectious Disease. Williams and Wilkins Company, Baltimore, 1962.
15. Burrill, D.Y. Effect of Orthodontic Treatment on Caries Susceptibility. *J. Dent. Res.* 20:253-254 (June), 1941.
16. Collee, J.G., Watt, B., Fowler, E.B., and Brown, R. An Evaluation of the GasPak System in the Culture of Anaerobic Bacteria. *J. Appl. Bacteriol.* 35:71-82, 1969.
17. Dobell, C. Antony van Leeuwenhoek and his "Little Animals". Harcourt, Brace and Company, New York, 1932.
18. Dolce, J.J. Caries Incidence in Relation to Orthodontic Therapy. *A.J.O.* 36:534-545 (July), 1950.
19. Dwyer, D.M., and Socransky, S.S. Predominant Cultivable Microorganisms Inhabiting Periodontal Pockets. *Brit. Dent. J.* 124:560-564, 1968.
20. Edwardsson, S. Characteristics of Caries-Inducing Human Streptococci Resembling Streptococcus mutans. *Arch. Oral Biol.* 13:637-646, 1968.
21. Ellner, P.D., Granato, P.A., and May, C.B. Recovery and Identification of Anaerobes: A System Suitable for the Routine Clinical Laboratory. *Appl. Microbiol.* 26:904-913, 1973.
22. Fauchard, P. The Surgeon Dentist. Trans. from Second Edition, 1746, by L. Lindsay. Butterworth, London, 1946.
23. Fitzgerald, R.J., and Keyes, P.H. Demonstration of the Etiologic Role of Streptococci in Experimental Caries in the Hamster. *J.A.D.A.* 61:9-19, 1960.

24. Fitzgerald, R.J., Jordan, H.V., and Archad, H.O. Dental Caries in Gnotobiotic Rats Infected with a Variety of Lactobacillus acidophilus. Arch. Oral Biol. 11:473-476, 1966.
25. Fitzgerald, R.J., Jordan, H.V., Stanley, H.R., Poole, W.L., and Bowler, A. Experimental Caries and Gingival Pathologic Changes in the Gnotobiotic Rat. J. Dent. Res. 39:923-935, 1960.
26. Florestano, H.J. Acidogenic Properties of Certain Oral Microorganisms. J. Dent. Res. 21:263, 1942.
27. Gibbons, R.J., and Banghart, S.B. Synthesis of Extracellular Dextran by Cariogenic Bacteria and Its Presence in Human Dental Plaque. Arch. Oral Biol. 12:11-25, 1967.
28. Gibbons, R.J., Berman, K.S., Knoettner, P., and Kapsimalis, B. Dental Caries and Alveolar Bone Loss in Gnotobiotic Rats Infected with Capsule Forming Streptococci of Human Origin. Arch. Oral Biol. 11:549-560, 1966.
29. Gibbons, R.J., and Socransky, S.S. Intracellular Polysaccharide Storage by Organisms in Dental Plaques. Arch. Oral Biol. 7:73-80, 1962.
30. Gibbons, R.J., Socransky, S.S., Sawyer, S., Kapsimalis, B., and MacDonald, J.B. The Microbiota of the Gingival Crevice Area of Man - II The Predominant Cultivable Organisms. Arch. Oral Biol. 8:281-289, 1963.
31. Goadby, K.W. Microorganisms in Dental Caries. J. Brit. D. A. 21:65, 1900.
32. Gordon, D.F., Stutman, M., and Loesche, W.J. Improved Isolation of Anaerobic Bacteria From the Gingival Crevice Area of Man. Appl. Microbiol. 21:1046-1050, 1971.
33. Holdeman, L.V., and Moore, W.E.C. Anaerobe Laboratory Manual, Second Edition, V.P.I. Anaerobe Laboratory, Blacksburg, Virginia, 1973.
34. Jay, P. An Anaerobe Isolated from Dental Caries. J. Bact. 14:385, 1927.

35. Jay, P., and Voorhees, R.S. B. acidophilus and Dental Caries. D. Cosmos 69:977, 1927.
36. Jordan, H.V., and Keyes, P.H. In Vitro Methods for the Study of Plaque Formation and Carious Lesions. Arch. Oral Biol. 11:793-801, 1966.
37. Kelstrup, J. The Incidence of Bacteroides melaninogenicus in Human Gingival Sulci and Its Prevalence in the Oral Cavity at Different Ages. Periodontics 4:14-18, 1966.
38. Keyes, P.H. Infectious and Transmissible Nature of Experimental Dental Caries. Arch. Oral Biol. 1:304-320, 1960.
39. Keyes, P.H. Questions Raised by the Infections and Transmissible Nature of Experimental Caries. Conf. on Oral Biol. J. Dent. Res. 39:1086, 1960.
40. Kligler, L.J. A Biochemical Study and Differentiation of Oral Bacteria with Special Reference to Dental Caries. J. Allied D. Soc. 10:141-166, 1915.
41. Krasse, B. The Effect of Caries-Inducing Streptococci in Hamsters Fed Diets with Sucrose or Glucose. Arch. Oral Biol. 10:223-226, 1960.
42. Krasse, B. Human Streptococci and Experimental Caries in Hamsters. Arch. Oral Biol. 11:429-436, 1966.
43. Loesche, W.J. Oxygen Sensitivity of Various Anaerobic Bacteria. Appl. Microbiol. 18:723-727, 1969.
44. Loesche, W.J., and Syed, S.A. The Predominant Cultivable Flora of Carious Plaque and Carious Dentin. Car. Res. 7:201-216, 1973.
45. Martin, W.J. Practical Method for Isolation of Anaerobic Bacteria in the Clinical Laboratory. Appl. Microbiol. 22:1168-1171, 1971.
46. Miller, W.D. Fermentation in the Human Mouth: Its Relation to Caries of the Teeth. The Independent Practitioner 2:57-65, 1884.

47. Miller, W.D. The Micro-Organisms of the Human Mouth. The Local and General Diseases Which are Caused by Them. S.S. White Dental Mfg. Co., Philadelphia, 1890.
48. Miller, W.D. New Theories Concerning Decay of Teeth. D. Cosmos, 47:1293-1301, 1905.
49. McCabe, R.M., Keyes, P.H., and Howell, A. An In Vitro Method for Assessing the Plaque-forming Ability of Oral Bacteria. Arch. Oral Biol. 12:1653-1656, 1969.
50. McMinn, M.T., and Crawford, J.J. Recovery of Anaerobic Microorganisms from Clinical Specimens in Pre-Reduced Media versus Recovery by Routine Clinical Laboratory Methods. Appl. Microbiol. 19:207-213, 1970.
51. Noyes, H.J. Dental Caries and the Orthodontic Patient. J.A.D.A. 24:1251 (Aug.), 1937.
52. Orland, F.J., Blayney, J.R., Harrison, R.W., Reyniers, J.A., Trexler, P.C., Ervin, R.F., Gordon, H.A., and Wagner, M. Experimental Caries in Germ-free Rats Inoculated with Enterococci. J.A.D.A. 50:259-272, 1955.
53. Orland, F.J., Blayney, J.R., Harrison, R.W., Reyniers, J.A., Trexler, P.C., Wagner, M., Gordon, H.A., and Luckey, T.D. Use of the Germ-free Animal Technic in the Study of Experimental Dental Caries I. Basic Observations on Rats Reared Free of All Microorganisms. J. Dent. Res. 33:147-174, 1954.
54. Owen, O.W. A Study of Bacterial Counts (Lactobacilli) in Saliva Related to Orthodontic Appliances. Am. J. Orthodont. 35:672, 1949.
55. Pinner, B., Pinner, M., and Krause, A.K. Robert Koch The Aetiology of Tuberculosis (Translation), Am. Rev. Tuberc. 25:285, 1932.
56. Priny, H. Dental Chronology: A Record of the More Important Historic Events in the Evolution of Dentistry. Lea and Febiger, Philadelphia, 1945.
57. Riggs, J.R. A Study of Acidogenesis by the Microflora of Dental Plaque in Orthodontic Patients. Master's Thesis, Loyola University, 1969.

58. Rosebury, T. Microorganisms Indigenous to Man. McGraw-Hill Book Co., Inc. New York, 1962.
59. Schafer, W.G., Hive, M.K., and Levy, B.M. A Textbook of Oral Pathology. W.B. Saunders Co., Philadelphia and London, 1963.
60. Society of American Bacteriologists. Manual of Microbiological Methods. McGraw-Hill Book Co., Inc. New York, 1957.
61. Socransky, S.S., Gibbons, R.J., Dale, A.C., Bortnick, L., Rosenthal, E., and MacDonald, J.B. The Microbiota of the Gingival Crevice Area of Man - I. Total Microscopic and Viable Counts and Counts of Specific Organisms. Arch. Oral Biol. 8:275-280, 1963.
62. Sutter, V.L., Attebery, H.R., Rosenblatt, J.E., Bricknell, K.S., and Finegold, S.M. Anaerobic Bacteriology Manual. U.C.L.A., Los Angeles, 1972.
63. Underwood, A.S. On the Influences of Microorganisms in the Production of Caries. Am. J. D. Sci. 18:83-87, 307-317, 372-375 (July), 1884.
64. Volker, J.F. Relation of Oral Biochemistry of Sugars to the Development of Caries. J.A.D.A. 51:285-292, 1955.
65. Williams, N.B. The Effects of Sugars and Carbohydrates on Oral Flora. J.A.D.A. 51:278-284, 1955.
66. Willis, A.T. Anaerobic Bacteriology in Clinical Medicine. Butterworth Inc., Washington, D.C., 1964.

APPROVAL SHEET

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Oral Biology.

June 9, 1975
Date

John V. Madonia D.D.S., Ph.D.
John V. Madonia, D.D.S., Ph. D.